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Research report

Characterization and phosphorylation of CREB-like proteins in *Aplysia* central nervous system

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Abstract

Studies in *Aplysia californica* indicate that cAMP-mediated gene expression is necessary for long-term facilitation, a correlate of long-term memory. It has been shown that blocking the expression of cAMP-inducible genes in sensory neurons impedes long-term facilitation without any effect on short-term facilitation. Specifically, blocking the binding of CREB-like proteins or inhibiting the expression of a cAMP-inducible gene, C\EBP, impairs long-term facilitation. In this report, we show the presence of a family of CREB-like proteins in *Aplysia* CNS that specifically bind to the CRE sequence and cross-react with rat CREB antibodies. Similar to mammalian CREB proteins, *Aplysia* homologues interact with each other via leucine zipper domains. This interaction can be disrupted by peptides containing the CREB leucine zipper sequence. We demonstrate that a 43 kDa CREB-like protein present in CNS extracts can be phosphorylated in vitro by cAMP-dependent protein kinase A. Moreover, exposure of ganglia to serotonin (5-HT), a transmitter involved in long-term facilitation, increases the phosphorylation of this protein. This biochemical data further supports the involvement of CREB-like proteins in memory storage.

Keywords: Long-term memory; CREB; cAMP; *Aplysia*

1. Introduction

Studies of sensitization, a simple form of non-associative learning, of the siphon-elicited siphon-gill withdrawal and tail-elicited tail-siphon withdrawal reflexes in *Aplysia californica* has revealed some of the molecular mechanisms of short- and long-term memory for these reflexes [6,20,32,35]. Memory for sensitization has been shown to be stored in a distributed fashion in both mono- and polysynaptic pathways [8,19]. One locus for this memory is the monosynaptic connection between the sensory and the corresponding motor neurons [8,53]. This monosynaptic component shows both short- and long-term facilitation of transmitter release which can be associated with learning-related changes in behavior [7–9,18]. In intact animals, a single sensitizing electrical stimulus to the tail produces short-term behavioral enhancement that lasts for minutes to hours while five or more stimuli results in long-term behavioral enhancement that lasts for days to weeks [18,46,54]. The short-term memory for sensitization in-

volves enhancement of transmitter release from sensory neurons, which occurs by means of second messenger-mediated protein phosphorylation [9,10,28,51]. In contrast, the long-term memory for sensitization requires gene expression and protein synthesis, and involves the growth of new synaptic connections and increased transmitter release at sensory-motor neuron synapses [11,22,44].

A large body of evidence shows that serotonin (5-HT) is one of the transmitters involved in memory storage in *Aplysia* [15,21,34,37]. Application of 5-HT increases intracellular cAMP concentrations in sensory neurons and can produce both short- and long-term facilitation of the sensory-motor neuron synapses [4,43,45]. Prolonged exposure of sensory neurons to cell-permeable analogues of cAMP or 5-HT also produces long-term facilitation and the associated morphological changes, both of which have been shown to require gene expression and protein synthesis [38,44,45]. Specifically, the expression of genes containing the CRE (Ca^{2+}/cAMP response element) sequence have recently been implicated in long-term facilitation of sensory-motor neuron synapses [1,12].

The transcription factor CREB (cAMP/calcium response element binding protein) is a member of the bZIP

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(basic leucine zipper) family of proteins that mediates gene expression in response to increases in intracellular cAMP or Ca^{2+} [16,27,29,39,48,39,55]. When phosphorylated either by cAMP-dependent protein kinase A (PKA) or calcium/calmodulin-dependent protein kinase (CaMK). CREB can induce the expression of CRE-containing genes [13,16,23,48]. These include transcription factors (e.g. c-*fos*), neuropeptides (e.g. enkephalin, somatostatin), biosynthetic enzymes (e.g. tyrosine hydroxylase) and growth factors (e.g. VGF) [for reviews see [40],[47]]. Recent studies indicate that CREB is involved in long-term memory storage in both vertebrates and invertebrates [17]. For example. "knockout" mice lacking the CREB gene show normal short-term memory (up to 60 min) but impaired long-term memory when tested 2 or 24 h after training [5]. When an inducible blocker of CREB (a CREB isoform which blocks the induction of CRE-containing genes) was expressed in *Drosophila* prior to training in a classical conditioning paradigm, long-term memory was severely disrupted [56]. Also, over-expression of wild type CREB facilitated the long-term memory formation for this paradigm [57]. In *Aplysia*, the expression of a reporter gene containing a CRE sequence is enhanced in sensory neurons following repeated applications of 5-HT [30]. Blocking the expression of Ca^{2+}/cAMP-inducible genes by injection of CRE-containing oligonucleotides into sensory neurons specifically impedes long-term facilitation without any effect on short-term facilitation [1,12]. Moreover, when the expression of the CRE-containing immediate-early gene C/EBP (CAAT-element binding protein) is inhibited, 5-HT-mediated long-term facilitation is blocked [1]. These studies show that the induction of CRE-containing genes is critical in establishing long-term facilitation in *Aplysia*.

In this report, we examined some of the biochemical properties of CREB-like proteins present in *Aplysia* CNS. Using an antibody raised against the DNA-binding domain of rat CREB, we detected several CREB-like proteins in the *Aplysia* nervous system. Similar to mammalian CREB proteins, *Aplysia* homologues interact through leucine zipper domains and their binding to the CRE sequence can be disrupted by a CREB leucine zipper peptide. In addition, we show that a 43 kDa CREB-like protein is a substrate for PKA and is phosphorylated in intact ganglia following 5-HT treatment. The data presented in this manuscript further support the involvement of CREB in *Aplysia* memory storage.

2. Materials and methods

2.1. Materials

[^{32}P]ATP was purchased from Amersham and *Aplysia californica* were obtained from Alacrity (Redondo Beach, CA). CREB antibody 244 was generously provided by Dr. Marc Montminy, Salk Institute, San Diego.

2.2. Gel mobility shift assay (GMSA)

GMSA using *Aplysia* CNS extracts were carried out essentially as described previously [12]. A double-stranded oligonucleotide containing the CRE sequence (5'-GCC-CTCCTTGGCTGACGTCAGAGAGAGAGTTCTGCA-3' and its complementary sequence) from the rat somatostatin gene [39] was radioactively labeled using T4 polynucleotide kinase (New England Bio Lab.) and [^{32}P]ATP as described previously [14]. The specific binding of this CRE-sequence containing probe to *Aplysia* CREB-like proteins has been previously characterized [12]. The probe was purified in an acrylamide gel. The radioactive probe was incubated with protein extracts from *Aplysia* CNS which were prepared as described previously [12]. The oligonucleotides bound to the proteins were separated from free oligonucleotides in a low ionic strength 6% polyacrylamide gel. The migration of the DNA-protein complex was detected by autoradiography of the dried gel. For super-shift or peptide competition assays, the CNS extract was pre-incubated for 15 min with antibodies or leucine zipper peptides prior to the addition of the probe. For competition assays, the following leucine zipper peptides were used:

CREB: NH$_2$-EYVKCLENRVAVLENQNKTLIEELKA-
       LKDLYCHKS-COOH

Jun: NH$_2$-RIARLEEKVKTLKAQNSELASTANM-
       LREQVAQLKQK-COOH

Fos: NH$_2$-ELTDTLQAETDQLEDEKSALQTEIAN-
       LLKEKEKLEF-COOH

nMyc: NH$_2$-YVHSLQAEEHQLLLEKEKLQARQQQ-
       LLKKIEH-COOH.

2.3. Protein purification

Aplysia CNS was homogenized in a buffer containing 50 mM Tris-HCl (pH 7.8), 10 mM KCl, 0.2 mM EDTA, 0.5 mM DTT and the protease inhibitors PMSF, aprotinin, pepstatin, and benzamidine. Glycerol (final concentration 10%) was then added, followed by the addition of KCl (0.6 M final concentration). The mixture was rotated gently at 4°C to dissociate proteins bound to the DNA. At the end of the incubation, the mixture was centrifuged at $14\,000 \times g$ for 30 min. The supernatant was diluted to a salt concentration of 0.42 M and loaded onto a DEAE cellulose column equilibrated with the same buffer. Under these high salt conditions, the DNA bound to the DEAE matrix. The flow-through solution, containing the protein, was collected and diluted to 0.1 M KCl. *E. coli* DNA (final concentration 10 μg/ml) was added and the solution was incubated at 4°C for 30 min. The mixture was then loaded on a CRE-oligonucleotide affinity column. The affinity matrix was prepared by cross linking the oligonucleotides to cyanogen bromide (CNBr) activated Sepharose [31]. The nonspecifically bound proteins were

eluted by washing the column with the Tris buffer containing 0.3 M KCl. The tightly bound CREB protein was eluted by increasing the salt to 0.6 M KCl. GMSAs were performed throughout the purification procedure to monitor CREB binding activity. Bovine brain CREB was partially purified using a CRE-affinity column [13]. Rat brain nuclear extracts were prepared as described previously [49].

2.4. Western blots

Protein extracts were boiled in sample buffer for 3 min and run on a 8.5% SDS PAGE gel. The proteins were then transferred to Immobilon-P paper using a semi-dry transfer apparatus (Millipore). Immunoreactivity was detected using an alkaline phosphatase kit (Promega) as described by the vendor.

2.5. In vitro phosphorylation and immunoprecipitation

Aplysia protein extracts were incubated with 0.1 μg of the catalytic subunit of protein kinase A in the presence of 2 μM [^{32}P] ATP in a buffer consisting of 50 mM Hepes pH 7.4, 10 mM $MgCl_2$, and 1 mM EDTA for 30 min at 30°C. The phosphorylation reaction was terminated by the addition of SDS to a final concentration of 0.5%. The CREB protein was immunoprecipitated using anti-rat CREB antibody 244. The entire immunoprecipitation procedure was carried out at 4°C. The SDS extracts were adjusted to contain (final concentration) 300 mM NaCl, 50 mM NaF, 14 mM EDTA, 10 mM sodium phosphate pH 7.4 and 1.8% NP-40 [25] followed by centrifugation at 14 000 × g for 10 min. The high concentration of salt and detergent were used to reduce non-specific adsorption during the precipitation procedure, and the NaF and EDTA are used to inhibit phosphatase activity present in the serum. The control and the experimental extracts were pre-cleared with 20 μl of protein A agarose (BRL) for 30 min at 4°C to remove any non-specifically bound proteins. After centrifugation for 2 min at 500 × g, the supernatant solutions were incubated with 0.5 μg of CREB antiserum and 10 μl of protein A agarose overnight at 4°C. To remove non-specifically bound proteins, the protein–antibody complex bound to the agarose beads were washed 3 times in RIPA buffer (50 mM Tris HCl (pH 8.0), 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, and 0.1% SDS) and once with 50 mM Tris-HCl (pH 7.5) + 0.1% NP40. The pelletized material was boiled in sample buffer for 3 min and the phosphoproteins analyzed on a 10–16% SDS gradient gel.

2.6. Back-phosphorylation

Aplysia were anesthetized by injecting one-half the body weight of ice-cold isotonic $MgCl_2$ (363 mM). The pleural and pedal ganglia were dissected and placed in L15

culture medium. The ganglia from one side were used as controls and their symmetric partners were used as experimentals. The experimental ganglia were treated with 20 μM 5-HT and 0.1 mM isobutylmethylxanthine (IBMX) while the control ganglia remained in culture medium for 30 min at room temperature. Following the treatment, the ganglia were quickly homogenized in 20 mM citric acid pH 2.8 and 0.01% NP40 as described previously [25]. Citric acid extraction has been shown to inhibit the endogenous protein kinase and protein phosphatase activities [42]. The homogenates were centrifuged and the supernatant solutions were neutralized with sodium phosphate buffer. Equal amounts of protein from control and experimental samples were phosphorylated using 0.5 μg of the catalytic subunit of protein kinase A in the presence of 2 μM [^{32}P]ATP as described above. The reactions were terminated by the addition of a SDS solution to a final concentration of 0.5%. The CREB protein was immunoprecipitated as described above. The immunoprecipitated material was analyzed on a 10–16% gradient SDS gel. The incorporation of radioactivity was detected by autoradiography.
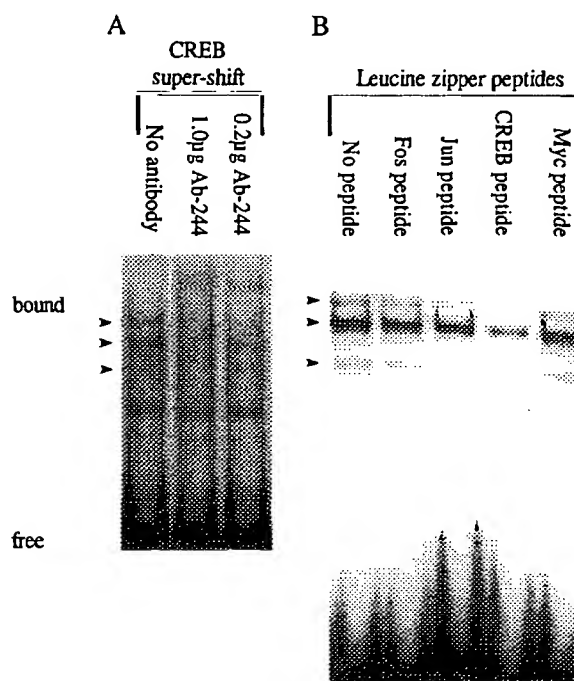

Fig. 1. Binding of Aplysia CREB-like proteins to CRE sequence. A. GMSA using Aplysia CNS extracts shows the presence of three specifically retarded bands (arrow heads). Pre-incubation of CNS extracts with antibody 244 causes super-shift of the upper most band. B. Inhibition of gel-retardation by leucine zipper peptides. When the CNS extract is pre-incubated with CREB leucine zipper peptides, the intensities of the retarded bands are reduced suggesting the disruption of native CREB dimers. Fos or Myc zipper peptides did not affect dimer formation. The Jun leucine zipper peptide reduced the intensity of the lowest of the three retarded bands suggesting the presence of Jun-like proteins in this band.

2.7. Generation of antibodies to the DNA-binding domain of the CREB protein

Approximately 5 mg of peptide 149 (NH$_2$-EEAARK-REVRLMKNREAARECRRKKKEYVK-COOH) was coupled to BSA using the amine cross-linker EDAC (Pierce) according to the protocol provided by the manufacturer. The conjugated peptide was injected into two rabbits as an emulsion with Freund's complete adjuvant. Each rabbit was boosted three times using Freund's incomplete adjuvant. The titer of the antibodies produced was determined using enzyme-linked immunoassays (ELISA). Rabbits were bled and serum collected when the titer of the antibodies was high. Initially, the crude serum was used in Western blots (using rat brain extracts) to identify the molecular weight of the cross-reacting band(s). For comparison, anti-CREB antibody 244 was also used. The CREB antibodies were then purified using a peptide affinity column. Since the peptide used for immunization contains a cysteine residue, a peptide affinity column was prepared using a Sulfo-link agarose resin (Pierce) according to the protocol provided by the manufacturer. The sulfo-linked coupling chemistry was utilized for purification of antibodies in order to remove any antibodies that might have been generated against the amino-cross linker region formed by the EDAC conjugation.

3. Results

3.1. Characterization of CREB-like proteins in Aplysia CNS using GMSA

We performed GMSAs using Aplysia CNS extracts and a radioactively-labelled oligonucleotide containing the CRE sequence as a probe. As reported previously using identical oligonucleotides, we detected three retarded bands (arrow heads Fig. 1) [12]. To characterize the composition of the retarded bands, super-shift and leucine zipper peptide competition assays were carried out. Fig. 1A shows the result of a gel super-shift assay using CREB antibody 244. The specificity of this antibody has been previously characterized [13,23,55]. When the Aplysia CNS extract was pre-incubated with either 0.2 or 1 μg of antibody 244, a super-shift of only the top band was seen. We next used peptides containing the leucine zipper domain to further characterize the retarded bands. Pre-incubation of Aplysia CNS extracts with the CREB leucine zipper peptide de-
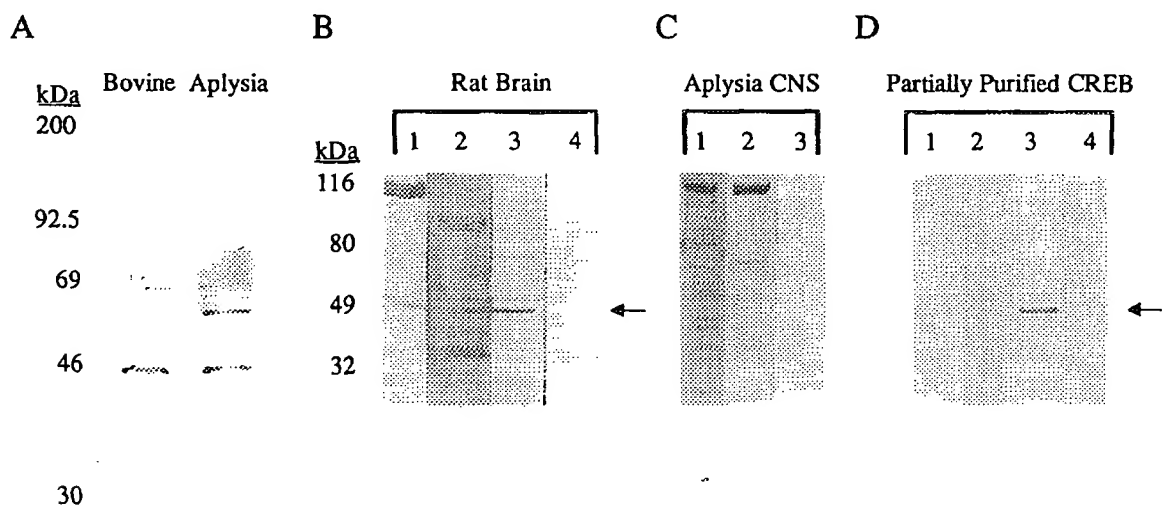

Fig. 2. Western blots of rat, Aplysia and bovine brain proteins. A. Partially purified CREB protein from bovine brain and Aplysia CNS were used in these experiments. A 43 kd protein in both experiments strongly cross-reacted with the anti-rat CREB antibody 244. Three other proteins also cross-reacted with the antibody in the Aplysia extract. However, only the 43 kd protein specifically bound to CRE oligonucleotides in Southwestern blots (data not shown). B. Western blots of rat brain protein using CREB antibodies. Lane 1 shows the binding of antibody 244 to the rat brain extract. Lane 2 shows the immunoreactivity of the crude serum containing the DNA-binding domain antibody. After affinity purification, the antibody cross-reacts with selective bands including the prominent CREB band (lane 3). Preabsorbing the affinity-purified antibody with peptide 149 abolishes the immunoreactivity (lane 4). The arrow indicates the migration of the CREB protein. C. Western blots of Aplysia CNS extracts using the DNA-binding domain antibodies. Lane 1 shows the immunoreactivity of the crude serum containing the DNA-binding domain antibodies. The affinity-purified antibody cross-reacts with several Aplysia CRE-binding proteins (lane 2). The pre-incubation of the affinity-purified antibodies with peptide 149 precludes the specific immunoreactivity (lane 3). D. Western blots of partially purified Aplysia and bovine brain CREB using the DNA-binding domain antibodies. The cross-reacting bands of the partially purified Aplysia CREB using the affinity-purified antibodies (lane 1) and the affinity-purified antibodies after preabsorption with peptide 149 (lane 2). The cross-reactive bands of the partially purified bovine CREB using the affinity-purified antibodies (lane 3) and the affinity-purified antibodies after preabsorption with peptide 149 (lane 4). The arrow indicates the migration of the CREB protein.

creased the intensities of all three retarded bands (Fig. 1B). Incubation with Fos or Myc leucine zipper peptides had no effect. However, the Jun leucine zipper peptide reduced the intensity of the lowest of the three retarded bands.

3.2. Inhibition of GMSA by the DNA-binding domain antibody

To confirm that the proteins present in the retarded bands have similar DNA-binding domains as mammalian CREB, GMSAs were carried out using antibody 149 (raised to DNA-binding domain of rat CREB) (Fig. 3). In the absence of protein extracts, no retarded bands were seen (lane 1). Specifically retarded bands were detected using *Aplysia* CNS extract (lanes 2). When the protein extract was pre-incubated with 0.2 μg, 0.4 μg and 0.8 μg of the affinity-purified antibody prior to the addition of the CRE probe, the binding was inhibited (lanes 4, 5 and 6 respectively). Pre-incubation of the CNS extract either with antibody storage buffer (lane 3), or preimmune serum (data not shown) did not affect the binding.

3.3. Identification of CREB-like proteins using Western blots

In order to identify the presence of CREB-like proteins in *Aplysia* CNS, Western blots using anti-rat CREB antibody 244 and 149 were used. No specific immunopositive band(s) were observed in Western blots using crude protein extracts and antibody 244 possibly due to the low abundance of *Aplysia* CREB. We proceeded to enrich the CREB protein as described in the Materials and Methods section and then used this partially purified extract for Western blotting. Antibody 244 cross-reacted with a 43 kDa protein which comigrates with the bovine brain CREB protein (Fig. 2A). There were three other proteins in the partially purified *Aplysia* preparation which also cross-reacted with the antibody. However, when the samples were analyzed using [^{32}P]-labelled CRE-containing oligonucleotides in a Southwestern blot, only the 43 kDa band was detected (data not shown).

Antibody 244 was raised using the rat CREB sequence N-terminal to the DNA-binding domain [23]. This antibody is unlikely to cross-react with all members of the CRE-binding protein family. In order to identify other CRE-binding proteins present in *Aplysia* CNS, we utilized an antibody raised to the DNA-binding domain of rat CREB (antibody 149). Fig. 2B shows that the crude serum cross-reacts with several proteins present in a rat brain nuclear extract (lane 2). After affinity purification, the antibody cross-reacted with 6–8 bands (lane 3). The most intensely stained band (lane 3) corresponds to the migration of CREB protein as detected by antibody 244 (lane 1). When the purified antibody was pre-incubated with peptide 149, no immunopositive bands were detected (lane 4). Fig. 2C shows that when *Aplysia* extracts were used, several pro-
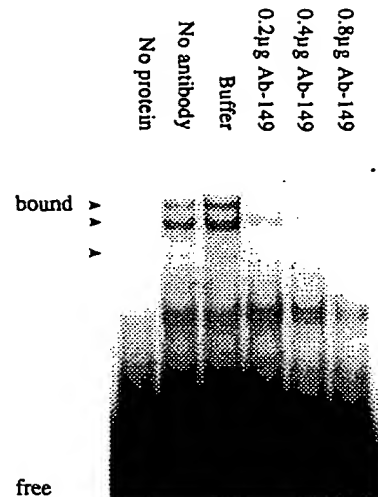

Fig. 3. Inhibition of GMSA by the DNA-binding domain antibody. The figure shows the picture of a GMSA autoradiograph using *Aplysia* CNS extracts and a radioactive CRE probe. Lane 1 shows the migration of the CRE probe in the absence of any protein extract. Lane 2 shows that three retarded bands are detected in the presence of *Aplysia* CNS extracts (arrow heads). Addition of 0.8 μl of PBS (antibody storage buffer) did not affect binding (lane 3). Lane 4–6 shows that incubation of *Aplysia* CNS extracts with increasing concentrations (0.2 μg, 0.4 and 0.8 μg) of the affinity-purified DNA-binding domain antibody (in PBS) inhibits gel-retardation.

teins cross-reacted with the crude serum (lane 1). When the affinity-purified antibody was used, eight bands were detected (lane 2) including a less intensely stained band migrating around 43 kDa. These bands were not detected after preabsorbing the antibody with peptide 149 (lane 3). To detect low abundance CRE-binding proteins, *Aplysia* CNS extracts were partially purified using a CRE-oligonucleotide affinity column. Using this partially purified extract, we detected two bands migrating around 43 kDa that cross-reacted with the affinity-purified antibody (Fig. 2D, lane 1). The upper band comigrates with partially purified bovine brain CREB protein (lane 3). Both the *Aplysia* (lane 2) and the bovine brain (lane 4) CREB proteins were not detected when the antibody was pre-incubated with peptide 149 prior to use.

3.4. In vitro phosphorylation of Aplysia CREB-like protein by PKA

To determine if *Aplysia* CREB-like proteins can be phosphorylated by the cAMP-dependent protein kinase A (PKA), partially purified CREB protein was used for in vitro phosphorylation. The protein extract was phosphorylated using the catalytic subunit of PKA and immunoprecipitated as described in the Materials and Methods. Fig. 4 shows that after PKA phosphorylation, a 43 kDa phosphoprotein was immunoprecipitated (lane 2). In the absence of antibody or kinase, no signal was detected (lane 1 and data not shown).
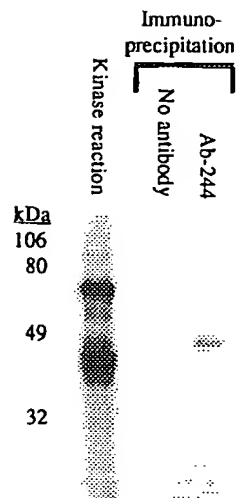
Fig. 4. In vitro phosphorylation of CREB-like protein by cAMP-dependent protein kinase A. The partially purified *Aplysia* protein (same extract as in Fig. 2) was phosphorylated in vitro using the catalytic subunits of protein kinase A. Lane 1 shows the amount of phosphoproteins present in the kinase reaction after treatment with protein kinase A. A 43 kd phosphoprotein was precipitated using CREB antibody 244 (lane 3). Control experiments without the antibody (lane 2), the protein extract, or the kinase did not precipitate the 43 kd protein.

3.5. 5-HT treatment increases the phosphorylation of a CREB-like protein in Aplysia ganglia

The phosphorylation of *Aplysia* CREB-like proteins in pedal-pleural ganglia following prolonged 5-HT application was next examined. Fig. 5 shows a representative picture of a back-phosphorylation experiment. Phospho-
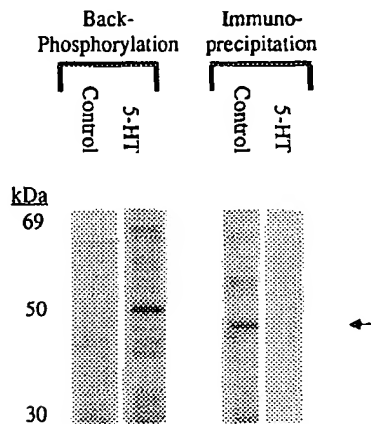

Fig. 5. Increased phosphorylation of CREB protein in *Aplysia* central nervous system following 5-HT treatment. Back-phosphorylation was used to visualize the degree of CREB phosphorylation. The precipitated materials were separated on a 10–16% SDS gel and the autoradiograph of the gel is shown. The arrows point to the migration of the CREB protein. Incorporation of radioactive phosphate in the CREB band is significantly less compared to the control sample. This suggests the CREB protein in the experimental sample was already phosphorylated by the 5-HT treatment.

rylation of proteins during 5-HT treatment would result in less [^{32}P] incorporation during subsequent back-phosphorylation by PKA. Fig. 5A shows the incorporation of radioactivity during the back-phosphorylation reaction in a control and 5-HT treated ganglia. Following immunoprecipitation with antibody 244, a 43 kDa CREB-like protein was detected (Fig. 5B, arrow). The amount of [^{32}P] incorporated in the 43 kDa protein from the experimental sample was lower when compared to the control sample. Control experiments showed that the immunoprecipitation of this protein can be blocked by pre-incubating the antibody with the synthetic peptide used for immunization (data not shown).

4. Discussion

Memory is initially stored in a transient state and later converted into more long-lasting forms [50]. The process of conversion of short-term memory into long-term memory is called *consolidation* and probably involves more than one mechanism. Studies in *Aplysia*, as well as in *Drosophila*, mice and rats indicate a unifying theme for consolidation of memory [1,5,12,56,57]. These studies show that in both invertebrates and vertebrates, cAMP-mediated gene expression is involved in the formation of long-term memory. In all of the cases, CREB appears to be necessary for memory consolidation. Although CREB has been implicated in *Aplysia* memory, the protein has not yet been isolated nor has its biochemical properties been characterized. In this report, we present biochemical evidence to corroborate the presence of and characterize CREB-like proteins in *Aplysia* CNS. Furthermore, we show that a 43 kDa CREB-like protein could be phosphorylated in vitro by the catalytic subunit of PKA and by application of serotonin (5-HT) to intact ganglia.

CREB belongs to a family of transcription factors collectively known as basic-leucine zipper (bZIP) proteins (for review see [33]). Dimer formation via leucine zipper domains allows the basic regions to interact with the DNA enhancer sequence and is a requirement for transcriptional activity [52]. We examined the DNA binding and dimer formation properties of *Aplysia* CREB-like proteins using leucine zipper peptides and GMSA. As reported previously, we detected three specifically retarded bands using *Aplysia* CNS extracts [12]. The oligonucleotide probe used in the GMSA assays was identical to that used previously [12]. The binding of these retarded bands can be competed by other CRE sequence-containing oligonucleotides [12]. The CREB leucine zipper peptide specifically disrupts CREB dimer formation and inhibits transcription of CRE-containing genes when incubated with HeLa cell nuclear extract [14]. When added to *Aplysia* CNS extracts, this peptide specifically inhibited the binding of *Aplysia* CREB-like proteins to the CRE sequence (Fig. 1B). This is most likely due to the disruption of dimers by leucine

zipper peptides resulting in CREB monomer-peptide complexes which are unable to bind the CRE sequence with a strong affinity. Moreover, high concentrations of peptide were required to disrupt the dimer formation indicating either that the interaction between the native CREB monomers is strong or that there is not complete homology with the mammalian leucine zipper domain. It is interesting to note that the Jun leucine zipper peptide also reduced the intensity of the lowest of the three specifically retarded bands present in *Aplysia* CNS (Fig. 1B). This suggests the presence of heterodimers consisting of CREB-like and Jun-like proteins in this retarded band. Although not promiscuous, heterodimerization between related bZIP factors has been shown to take place. For example, CRE-binding factors such as ATF-2, ATF-3, and ATF-4 can heterodimerize with Fos and Jun [26,36]. This allows the cAMP/PKA pathway to interact with the diacylglycerol/PKC pathway at the transcriptional level. Further experiments will help clarify if these types of interactions are taking place in the *Aplysia* CNS.

In order to identify the proteins in *Aplysia* CNS which interact with the CRE sequence, we used antibody 149 which was raised against the DNA-binding domain of rat CREB. The affinity-purified antibody specifically cross-reacted in Western blots with several proteins present in *Aplysia* CNS extracts. Furthermore, when a partially purified extract was used, a 43 kDa protein was detected which comigrated with bovine brain CREB (Fig. 2D). When this antibody was used in GMSAs, the binding of all the specifically retarded bands was blocked. This inhibition is most likely caused by the interaction of antibodies to the DNA-binding domain resulting in a complex which is unable to cause retardation of the CRE probe. It is therefore likely that the proteins identified in the *Aplysia* CNS extract have similar DNA-binding domains as their mammalian counterparts. When antibody 244 was used for Western blotting, a 43 kDa protein was again detected in a partially purified *Aplysia* CNS extract (Fig. 2A). Moreover, inclusion of this antibody in a super-shift assay resulted in the altered migration of only the uppermost band (Fig. 1A). This suggest that the 43 kDa protein identified by Western blotting is a component of this retarded complex. However, this experiment cannot determine if both the monomers are bound to antibody 244.

Serotonin (5-HT)-mediated gene expression has been shown to be necessary for long-term facilitation at the sensory-motor neuron synapses in *Aplysia* [38]. The application of 5-HT increases the intracellular concentrations of cAMP in *Aplysia* sensory neurons [4,43]. Furthermore, prolonged application of 5-HT results in the translocation of the catalytic subunit of PKA into the nucleus which could induce the expression of CRE-containing genes [2]. Consistent with this, prolonged application of 5-HT to intact ganglia has been shown to induce the expression of a CRE/β-gal reporter gene injected into sensory neurons [30]. In mammalian systems, the induction of CRE-con-

taining genes has been shown to be due to the phosphorylation of CREB by PKA or CaMK [16,23,48,55]. It is therefore likely that 5-HT-mediated gene expression in *Aplysia* is mediated by CREB phosphorylation. To determine if *Aplysia* CREB is a substrate for PKA, in vitro phosphorylation assays using the catalytic subunit of PKA were carried out. Using a partially purified extract, we found that a 43 kDa phosphoprotein was immunoprecipitated by antibody 244. At present, however, we cannot identify which amino acid residue(s) is phosphorylated. Interestingly, the phosphorylation of this protein was also enhanced by the exposure of ganglia to 5-HT. This enhanced phosphorylation was detected using PKA-mediated back phosphorylation. This suggest that the site phosphorylated during back phosphorylation is likely to also be a PKA site in vivo. This experiment cannot rule out the involvement of other protein kinases in phosphorylating the CREB-like protein in response to 5-HT. In fact, it has been shown that mammalian CREB can be phosphorylated by PKA, CaMKII, CaMKIV or PKC on Ser133 [13,16,23,48]. Future experiments will help define the role of these kinases in CREB activation in *Aplysia*.

Sensory neurons, located in the abdominal and pleural ganglia, are key sites for plasticity of the siphon-elicited siphon-gill withdrawal and tail-elicited tail-siphon withdrawal reflexes [8,46]. Morphological changes, including increased varicosities, have been seen in these sensory neurons following behavioral training [3,24]. These long-term changes can be induced by cell permeable cAMP analogues possibly via CREB phosphorylation [41,45]. We have not yet specifically examined the phosphorylation of CREB-like proteins in the sensory neurons following cAMP or 5-HT application. Our preliminary experiments show no difference in gel-retardation pattern using either the total CNS extract or sensory neuron extracts. This suggests that the same CRE-binding proteins are present in the sensory neurons which may respond similarly to 5-HT application. Future experiments will help elucidate if 5-HT treatment or behavioral training increases the phosphorylation of CREB in sensory neurons, and whether phosphorylated CREB-like proteins are sufficient to cause the associated morphological changes.

Acknowledgements

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